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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Hevesi, et al.	Group Art Unit 1641
Appl. No.	:	09/833,030	
Filed	:	April 10, 2001	
For	:	METHOD FOR OBTAINING A SURFACE ACTIVATION OF A SOLID SUPPORT FOR BUILDING BIOCHIP MICROARRAYS	
Examiner	:	Tran, My-Chau T.	

DECLARATION UNDER 37 C.F.R §1.132

United States Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

Dear Sir:

1. This Declaration is being submitted to demonstrate the sensitivity, stability, reproducibility, and effectiveness of microarrays prepared using the methods of the present invention.

2. I am an inventor on the above-identified patent application and am familiar with the specification and prosecution history.

3. I have extensive technical experience in the field of the claimed invention as indicated in the attached Curriculum vitae provided herewith as Exhibit A.

4. Microarrays prepared according to the claimed methods provide enhanced sensitivity relative to commercially availabe products. As demonstrated in Figure 2 of the above-identified application, a spotting solution of 400nM on microarrays prepared according to the claimed methods yielded the same signal as a 2000nM solution on the commercially available products.

5. Microarrays prepared according to the claimed methods provide enhanced fixation capacity relative to commercially available products. As demonstrated in Example 3 of the above-identified application, the amount of nucleic acid which can be fixed to a given area of the

microarrays of the present invention is significantly greater than that which is fixed to the same area of commercially available products.

6. Microarrays prepared according to the claimed methods are stable when stored for significant periods of time.

Diaglass slides prepared according to the method of the present invention were stored at 2 different temperatures (4°C and 20°C-RT) and at 2 different storage conditions (under vacuum or not) for 6 months. After this period, biotinylated aminated DNA probes were spotted at different concentrations on stored slides (3 slides per storage condition) and on newly produced slides. The results are shown in Exhibit B for a spotted solution of 150nM probe concentrations. The biotinylated nucleotidic probes were then reacted with Cy3 streptavidin and the fluorescence present on the spots was quantified by a confocal scanner (Genetic Microsystem). After the revelation step, data have been quantified and compared to these obtained at the beginning of the study.

The yield of fixation of the probes was very high even after 6 months of storage in the 3 above described conditions, mainly at 4°C under vacuum, at 4°C without a vacuum and at room temperature (RT) under vacuum.

The stability study indicates that Diaglass Slides are stable for 6 months at 4°C (under vacuum or not) and for 6 months at RT under vacuum and the Diaglass Slides can be stored at 4°C and delivered at RT when packaged under vacuum.

7. The methods of the present invention enable microarrays to be manufactured with significant reproducibility from one batch to the next. The variability of the activation of microarrays prepared according to the claimed methods was tested over a period of six months by spotting of biotinylated aminated DNA probes and their quantification as described in the paragraph above. The table below shows the production date of the slides (batch) and the variability within the tested batches. The average variability within the produced microarrays over a six-month testing period was 12.62%.

Production date	Batch Variability (CV)
08/16/01	10.03%
08/31/01	15.21%
09/26/01	17.12%
10/02/01	10.70%
10/23/01	14.02%
10/31/01	12.06%

11/21/01	22.13%
12/07/01	3.07%
12/18/01	11.07%
01/21/02	8.81%
02/08/02	14.59%

Average : 12.62 %

This variability includes not only the chemistry but also the way the controls were performed to include the operator variability, the spotting variability and the revelation variability.

This demonstrates that the microarrays produced by the method of the present invention show a high reliability and low variability from batch to batch.

8. The claimed methods permit significant homogeneity to be obtained from one area of the slide to the next.

To test the homogeneity of functionalization, a biotinylated DNA concentration curve was spotted at 9 different places of a slide. The concentration of DNA ranged from 5nM spots to 150 nM spots. This spotting was repeated on 4 slides. After revelation with a streptavidin-cyanin 3 conjugate, signal intensities were quantified by fluorescence assay on a confocal scanner. The attached Exhibit C depicts the results for these 9 arrays present on one slide.

There was no significant difference observed between the 9 different arrays.

9. In addition to the ability to readily fix nucleic acids to the microarrays prepared according to the claimed invention, antibodies may also be efficiently fixed to the microarrays.

Antigens were spotted on the activated aldehyde glass slide (DIAGLASS slides, AAT) as follows:

The antigens were diluted to a final concentration of 100µg/ml in a spotting buffer Borate 0.05M pH 8, glycerol 15 %, NP40 0.02 % and spotted as an antigen at the surface of an aldehyde based polymer coated glass slide. The spotting was obtained with solid pins of 0.250 mm diameter and the spots were around 0.35 mm diameter final. After 4 washes of 2 minutes with blocking buffer for 1h30 at 20°C.

For detection of antibodies, the slides were incubated for 1h at 20°C with different human sera diluted to 1/100 in the blocking buffer. After 4 washes of one minute with a 10 mM maleate buffer containing 15 mM NaCl and 0.1% Tween pH 7.5 (washing buffer) slides were incubated for 45 min at 20°c with a conjugate of anti-human IgG(H+L)/gold particles of 10nm diameter (diluted 100 times) in 100 mM blocking buffer. Slides were washed 4 times (for 2 minutes) in the same washing buffer as before and then incubated for 10 min in the Silver Blue detection solution (EAT Namur) for obtaining the silver crystal precipitation. The slides were finally washed

in distilled water before being read in the scanner and quantified using Imachips software (WOW Company).

The antigens spotted on the slide were: La(SSN) Ag, JO-1 Ag, Scl-70 Ag, RNP/Sm Ag, Ro(SSA) Ag. Protein A gold was used as a positive control for detection, mouse antibody and streptavidin used as negative controls. The results are presented in Exibit D.

The results show that:

- For serum CH+::
    - A clear and very positive signal on the JO-1 antigen which signal is as high as the positive control of the protein A gold in the diffusion mode.
    - The signal intensity obtained for Scl-70 Ag is lower but is well detected by the diffusion method
- For serum 6768:
    - A clear and very positive signal on the JO-1 antigen which signal is close to the values of the positive control of the protein A gold in the diffusion mode.
    - A positive signal for the Scl-70 Ag and Ro(SSA)Ag which are well detected by the diffusion method

These results correlated with the ELISA methods performed in the clinical laboratory

9. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated : 7 September 2002

By: Professor José Remacle

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Exhibit A: Short CV

CURRICULUM VITAE

REMACLE, José, A. L.

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Rue de Bruxelles 61 5000 NAMUR Belgique
Tel : 32-(0)81-724123 (Office) Fax : 32-81-724135
Email : jrem@biocell.fundp.ac.be

DEGREES

Bachelor of Chemistry with maxima cum laude, 1970
Université Catholique de Louvain, Belgium.

Ph.D. in Sciences, Biochemistry, with maxima cum laude, 1973
Université Catholique de Louvain, Belgium.
Directeurs de thèse : Profs H. Beaufay and A. Trouet.
Laboratoire de Chimie Physiologique, Prof. C. de Duve.

POSITIONS

1970 - 1971 : *Junior research of the National Fondation for Scientific Research (F.N.R.S.)*
1971 - 1974 : *Research assistant of the F.N.R.S.*
1973 - 1974 : Fellowship of "Belgian American Educational Foundation" (Bourse C.R.B.)
1974 : Research fellowship of the European Molecular Biology Organization (E.M.B.O.)
1974 *Associate professor* Facultés Universitaires Notre-Dame de la Paix, Namur.
1980: *Professor* Facultés Universitaires Notre-Dame de la Paix, Namur
Director of the Laboratory of Cellular Biochemistry.
1985: *Full Professor, with tenure*
1992: *Visiting Scientist* University of Maryland, Baltimore County Campus

AWARDS

1968 : Prix de "l'Union Carbide European Research Associates"
1973 : Bourse William Hallam Tuck, of the fondation Francqui

1984 : Prix Vander Stricht de la Fondation André Vander Stricht
1992 : Senior Research Scholar at the University of Maryland, Baltimore for 1992-1995.

PROFESSIONAL EXPERIENCE

Research stage at the Rockefeler University, Prof. C. de Duve, from July to September 1973.

Post-doctoral research at the University of California, San Diego, U.S.A., from September 1973 to August 1974, in the laboratory of Prof. S. J. Singer.

Scientific mission of 4 months at the Biochemical Engineering Department of the University of Maryland in Baltimore,Laboratory of Prof G. Rao, in 1992.

Scientific mission at the Biochemical Engineering Department of the University of Maryland at Baltimore as Senior Research Scolar in March-April 1993.

SCIENTIFIC RESPONSABILITIES :

Head of the laboratory of cellular biochemistry and biology

Actual composition (1997)
7 PhDs in Science full research
12 PhDs Students
8 Graduate full research
8 Under-graduate students
7 technicians

Students and researchers already formed
Director of 16 PhDs Thesis passed from 1981 to 1997.
Director of 74 graduate students from 1974 to 1997.

RESEARCH CONTRACTS

<u>Research Contracts with Industries</u>

30 research contracts with Laboratoires Dausse, Synthélabo, Solvay-Biotec, Compagnie des développements agro-alimentaires (CDA), Kali-Chemie Pharma, La Floridienne, CELAC, laboratoires Oberval, Laboratoires Beaufour, UCB-Pharma, Lambdatech, Lipha, IPSEN, Zyma, Madaus Pharma, Servier.

Scientific Grants and Research contracts

14 contracts with the FNRS, FRFC,IRSIA, and Région Wallonne

PROFESSIONAL AND SCIENTIFIC ASSOCIATIONS

Member of 15 scientific societies

Member of 33 Ph.D. thesis jurys

Member of the research comitte for Biomed 1 and 2 of the EEC

PRESENTATIONS OF RESULTS IN SCIENTIFIC CONGRES

171.presentations in scientific meetings as author or co-author.

INVITED OR PLENARY CONFERENCES OR LECTURES.

75 presentations in scientific meetings under invitation

MAIN SUBJECTS OF RESEARCH

The Cellular Biochemistry and Biology Laboratory is working on cellular activation and interactions either in physiological conditions like ageing or in pathological situations in the vascular pathologies

Cellular Ageing mechanism has been investigated based on the in vitro model of cell culture. Modelisation of the ageing process has been possible using the thermodynamics of open systems; the role of Free radical and the importance of the antioxidant enzymeshave been deeply analysed.
Actual work is going on the molecular modulation of the genes expression during natural in vitro ageing and the stress induced ageing.The work compares the mRNA of specific genes overexpressed in old and stressed cells. It also investigates which signal transduction and transcriptional factor are activated durin the stress induced ageing

Study of endothelial cells under hypoxia in correlation with the development of varicose diseases.
We are studying the effects of hypoxia on endothelial cell metabolism either in vitro or in situ using umbilical and saphens perfused veins and the consequences of these modifications on the interactions between these cells and polymorphonuclear neutrophils and smooth muscle cells. The effect of phlebotonic drugs has been tested on cells in culture, on isolated organs and a clinical assay has been performed. This research is aimed to define the mechanism of varicose veins.

Development of new diagnostic assays using bioluminescence: ELISA, DNA probes for virus and bacteria detection.

Development of the biochips technology for the pharmaceutical, diagnostic and alimentation

PUBLICATIONS

The author's scientific output consists of 175 research papers in peer-reviewed international journals

Exhibit B: stability study

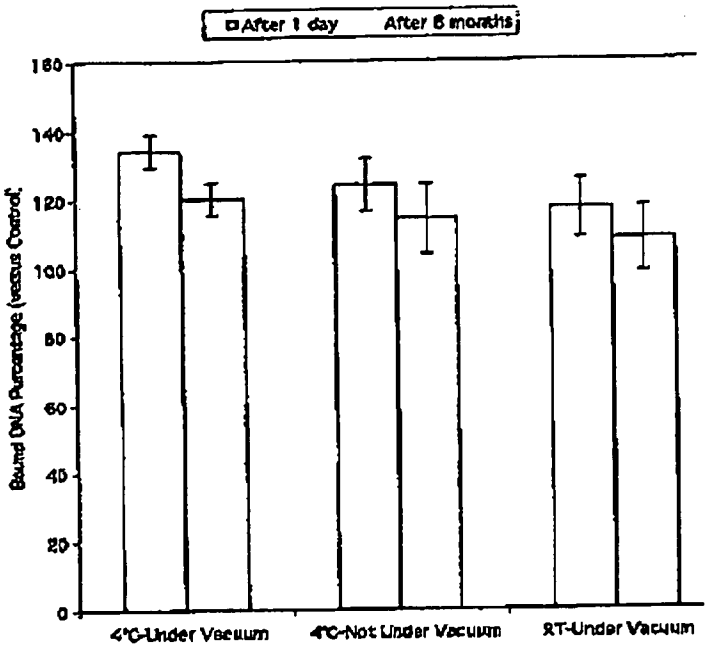

EXHIBIT C: Intra-batch Variability

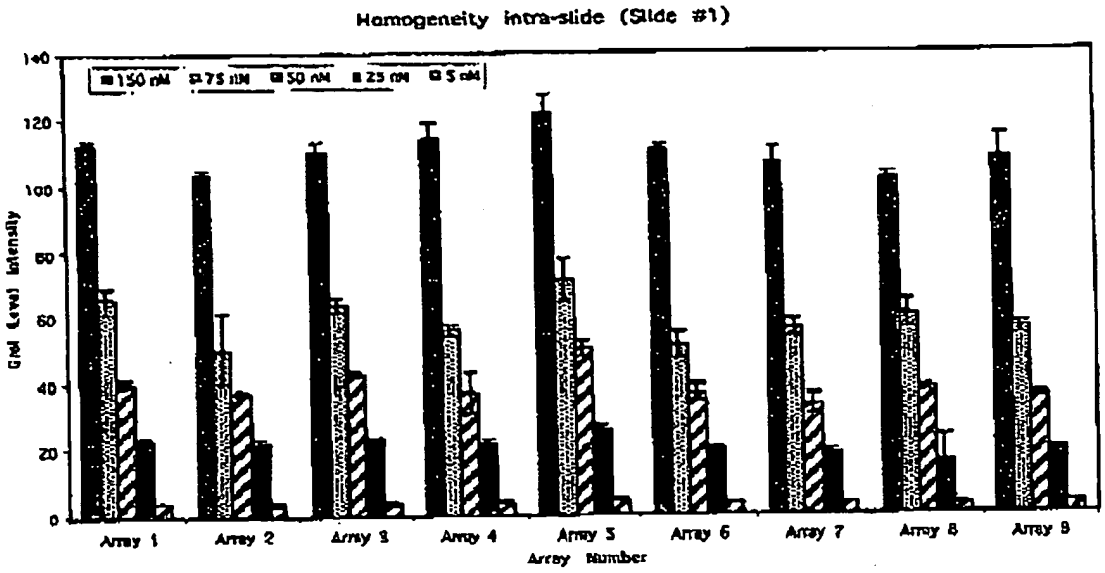

EXHIBIT D: Numerical results of autoimmune antibodies detection

Serum CH+	diffusion		transmissio n	
	mean	Std dev	mean	Std dev
protein A gold	35.768	0.798	29.425	0.109
Mouse antibody	-0.948	0.461	0.109	0.062
Streptavidin	-1.190	0.517	-0.484	0.222
La(SSB) Ag 1/2	-1.832	0.950	-0.378	0.504
JO-1 Ag 1/2	35.923	2.352	19.746	0.628
Scl-70 Ag 1/2	14.787	1.135	4.884	0.672
RNP/Sm Ag 1/2	-1.870	0.232	-0.296	0.181
Ro(SSA) Ag 1/2	-0.470	1.459	-0.192	0.431
protein A gold	27.983	2.756	26.198	1.306
buffers	-0.468	0.361	-0.412	0.750

Serum 6768	diffusion		transmission	
	mean	Std dev	mean	Std dev
protein A gold	54.243	4.186	42.260	2.334
Mouse antibody	-0.378	0.934	0.412	1.244
Streptavidin	-1.268	0.090	-1.505	1.451
La(SSB) Ag 1/2	11.277	2.166	2.581	0.207
JO-1 Ag 1/2	49.123	1.148	12.552	1.280
Scl-70 Ag 1/2	17.055	5.476	5.212	1.792
RNP/Sm Ag 1/2	-1.861	0.286	-0.455	0.348
Ro(SSA) Ag 1/2	38.668	2.042	8.694	1.012
protein A gold	50.779	2.908	37.181	2.016
buffers	-1.472	0.396	-0.335	1.080

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